Cross-Interactions of Bovine Chymotrypsinogen-A and Polyethylene Glycol

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By

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Abstract

As therapeutic proteins become increasingly important in medical research and pharmaceutical use, it is useful to understand the optimal conditions to facilitate crystallization, the most stable form for protein storage. The trial-and-error method is commonly used, but can be costly. Crystallization is dependent on the protein's interactions with itself and other components, and these interactions can be characterized by the second virial coefficient. This value can be experimentally determined and used to predict crystallization conditions, in a more systematic and cost-effective way. In this study, the interactions of the protein bovine chymotrypsinogen-A are studied with respect to the addition of polyethylene glycol (PEG), a polymer commonly used to stabilize therapeutic proteins. This study indicates a slight repulsive interaction between chymotrypsinogen-A and polyethylene glycol, and shows signs of the depletion effect of PEG, the presence of which causes the chymotrypsinogen-A to interact more strongly with itself.

Dedicated to my grandfathers, who brought me here

Acknowledgements

I want to thank my advisor, Dr. Michael Paulaitis, for guiding me through this project, and always having faith that I could complete it. Being in the Paulaitis group has been a challenge from the beginning, and one that has forced me to think in different ways. Dr. Paulaitis never stopped making me improve myself and change for the better.

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INTRODUCTION

1. Protein Crystallization

Historically, the costliest steps in producing proteins have been separation and purification, especially at the high purities required for pharmaceutical use. As of 2005, there were 230 biotechnology medicines and products on the market, with approximately 20 drugs approved a year.[1] In recent years, the global market for protein drugs has reached over $40 billion,[2] and more than 400 proteins are approved for clinical trials yearly, about half of which are monoclonal antibodies. In addition, the production of non-therapeutic proteins has a different set of process requirements[3] and product specifications, such as larger production quantities and more flexible purity specifications.

There are several factors that determine the approaches used to design protein separation processes.[3,4] The design methods for therapeutic applications are governed by four factors: small production scales, regulatory oversight, lower importance of manufacturing costs compared to those of clinical trials, and prompt process development. Many processes are designed through trial-and-error, often using results based on extrapolating from small-scale experiments, and different companies tend to use their own conventions when selecting their process operations, which may result in time-constrained inefficiencies. Monoclonal antibodies, which are increasingly being used and tested for pharmaceutical applications, also make use of similar approaches[4,5] for downstream

processing. As proteins are required on larger scales, and since manufacturing costs make up a large fraction of bulk protein production costs, a systematic process design method that creates a basis for initial product development could greatly benefit the US pharmaceutical industry. By bringing the design of protein separations to the same level of more mature chemical processes, we will advance both the large-scale high-purity separations required for non-therapeutic protein production, and process design methods applied prior to FDA certification that can improve product and process development for therapeutic applications.

The focus of this research project will be a specific bioseparations process development challenge: the bulk crystallization of proteins. Pharmaceutical proteins used in many therapeutic and non-therapeutic applications must meet certain stability conditions, including a long shelf life, even though proteins, in general, are less stable than the small organic molecules commonly used in such applications.[6] Since protein crystals are more stable than proteins in solution,[7] one approach to achieving long-term stability is to use crystallized proteins.

Proteins in aqueous solution can either precipitate or crystallize depending on the solution conditions, including pH, ionic strength, additives, temperature, and pressure. In addition, crystallization can lead to the formation of various types of crystals depending on the conditions. In the case of lysozyme, Velev et al.[8] observed absence of precipitation or crystallization, partial precipitation, compact crystals, and needle-like crystals, depending

on solution conditions. By altering conditions, such as acidity or ionic strength, or by adding different co-solvents to the solution, the protein-protein interactions can be modified, which, in turn, will affect the crystallization behavior.

2. Protein Interactions

Protein-protein interactions are quantified by the second osmotic virial coefficient, B_{ii}, which is the coefficient of the second term in the virial expansion of the equation for osmotic pressure, given below.

$$\beta\Pi = \frac{c_i}{M_i} + B_{ii}{c_i}^2 + \ldots \quad (1)$$

where Π is the osmotic pressure, β is the inverse of the thermal energy (product of the ideal gas constant and temperature of the system), c_i is the protein concentration, M_i is the protein molecular weight, and B_{ii} is the osmotic second virial coefficient. The osmotic pressure equation describes protein solution thermodynamics as deviations from ideal behavior, given by the first term on the right-hand side of equation (1). The second and higher order terms in the virial expansion account for the deviations from ideality. The osmotic second virial coefficient is related to the potential of mean force between two protein molecules in solution, and includes the effects of direct interactions between the two proteins, as well as indirect interactions through other protein molecules and the aqueous solution, including additives, such as polymers or salts.[9] If the protein-protein potential of mean force is attractive, B_{ii} is negative and the osmotic pressure is less than that for an ideal solution, while positive B_{ii} values correspond to repulsive potentials of mean force,[10] which lead to higher-than-ideal osmotic pressures.

According to the pioneering study done by George and Wilson[11] in 1994, proteins only crystallize in a small crystallization window of slightly negative osmotic second virial coefficients, or where protein-protein interactions are slightly attractive. For values of the osmotic second virial coefficient above (more positive than) the crystallization window, protein-protein interactions are repulsive, and the protein will remain in solution. For values of the osmotic second virial coefficient below (more negative than) the crystallization window, attractive protein-protein interactions dominate, and the protein will precipitate to form aggregates, rather than crystals. The osmotic second virial coefficient can be measured to predict the solution conditions designed to be within the crystallization window; identifying these conditions and how they can be achieved by adding alcohols and polyols is the focus of this thesis.

3. Effects of Additives

Only a few studies have been published on the effects of pH, ionic strength, and additives on a protein solution to determine their effects on protein crystallization. In a 2005 study done by Asthagiri et al.,[12] osmotic second virial coefficients for a lysozyme solution with no additives were measured as a function of the ionic strength. From this study, higher ionic strengths were found to increase attractive protein-protein interactions, resulting in a lower second osmotic virial coefficient and a less soluble protein. In another study, Liu et al.[13] used light scattering measurements of the osmotic second virial coefficient to determine the effects of alcohol additives, which are known to increase protein stability, on lysozyme solution were explored. In this case, it was found that adding alcohols

decreased protein-protein interactions, resulting in osmotic second virial coefficients that were more repulsive and enhanced lysozyme solubilities.

The goal of this research project is to study the effect of adding polyethylene glycol to aqueous solutions containing bovine chymotrypsinogen-A. Chymotrypsinogen is the inactive form of chymotrypsin, a pancreatic enzyme that is active toward the carboxyl groups of aromatic amino acids and catalyzes the cleavage of aromatic substances.[14] Chymotrypsinogen has a non-uniform surface charge that induces attractive electrostatic protein-protein interactions[8]. Polyethylene glycol (PEG) is a polymer that is commonly used to stabilize protein solutions, either as a simple additive or by attaching the polymer to proteins to prolong lifetime and increase potency.[15] In a 2002 study by Lu et al.,[16] PEG 4000 and PEG 1500 were reported to enhance chymotrypsinogen crystallization, or gelation at high concentrations of protein, indicating increased protein-protein attraction due to addition of PEG.

For a system with two components, the osmotic pressure is modeled as in Equation 2, where c_2 and M_2 are the concentration and molecular weight of the first component, c_3 and M_3 are the concentration and molecular weight of the second component, B_{22} and B_{33} are the self-interaction coefficients of each component, and B_{23} is the cross-interaction coefficient,

$$\beta\Pi = \frac{c_2}{M_2} + \frac{c_3}{M_3} + B_{22}{c_2}^2 + B_{33}{c_3}^2 + 2B_{23}c_2c_3 + \cdots \quad (2)$$

The cross-interaction coefficient is interpreted similarly to the osmotic second virial coefficient for a single component; a negative B_{23} reflects attractive intermolecular interactions between the two components, while a positive value reflects repulsive interactions.

4. Measurement Techniques

Several experimental techniques have recently been developed for measuring the cross-interaction virial coefficient, including cross-interaction chromatography and static light scattering. Cross-interaction chromatography is based on self-interaction chromatography and requires one component to be immobilized on to a chromatographic surface while the other component is eluted over it. The retention volume, which is the amount required to elute the solute from the column,[17] is measured in the chromatography process, and this value is used to calculate the retention factor. The retention factor can then be related to the cross-interaction virial coefficient. Several assumptions are made for cross-interaction chromatography with regards to the measured interactions; it is assumed that the eluting species only interacts with one immobilized particle at a time, and that the moving particles do not interact appreciably with each other.[18] The first assumption is dependent on the spacing of the immobilized component, and the second is valid at low concentrations,[17] which restricts this technique to a certain range of conditions. In addition, it is assumed that the interactions are the same regardless of the immobilization of one component.

Static light scattering measures the amount of light scattered by both the proteins and PEG in solution. The scattered light varies based on the distance between the molecules, and these distances change depending on the interactions between the particles. Traditional static light scattering is performed batchwise, and a separate sample must be made for every protein concentration. The light scattering at a certain angle is measured at a set of concentrations, and the correlation between the scatter and concentration can be used to determine the second virial coefficient.

The method used in this work is composition gradient static light scattering, which allows the samples to mix with a series of feed pumps, which can be controlled through a computer. This greatly facilitates the process, as only one stock sample is required, and the process becomes continuous. A schematic is given in Figure 1.

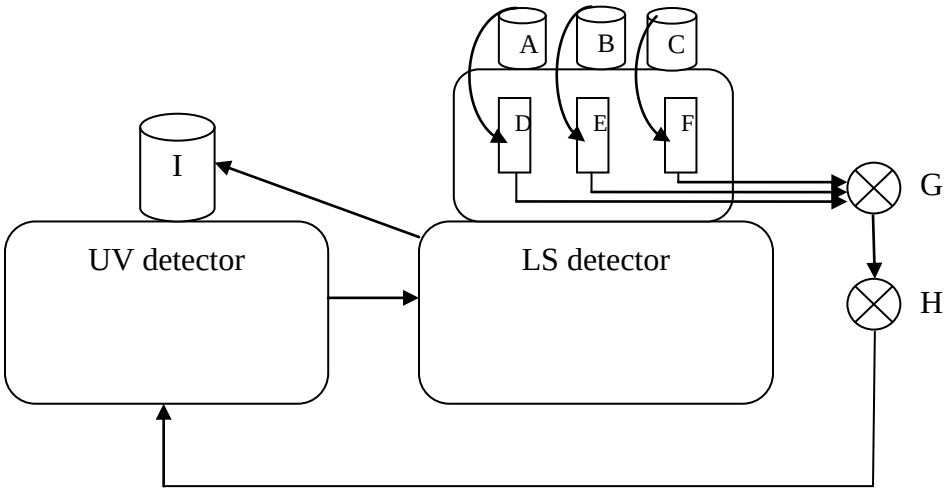

Figure 1: Composition Gradient Static Light Scattering Schematic – A,B,C: protein solution, buffer, and wash reservoirs respectively; D,E,F: syringe pumps; G: mixer; H: membrane filter; I: Waste; Arrows indicate direction of flow.

It should be noted that the signals from the detectors are sent to a computer to be analyzed, and analysis occurs in the same way as batch-style static light scattering.

EXPERIMENTS

1. Static Light Scattering Theory

The light scattering instrument consists of a laser light source and a photomultiplier that measures the amount of scattered light at a specific angle. From these measurements on a solution containing a single protein (no PEG), the osmotic second virial coefficient is obtained from the slope of a Zimm plot based on the following relation,

$$\frac{Kc_1}{R} = \frac{1}{M_1} + 2B_{11}c_1 \quad (3)$$

where c_1 is the concentration of the sample, M_1 is the protein molecular weight, K is an optical constant, and R is the excess Rayleigh ratio. The optical constant is calculated as follows:

$$K = \frac{4\pi^2 (dn/dc)^2 {n_0}^2}{N_A {\lambda_0}^4} \quad (4)$$

where dn/dc is the refractive index increment of the sample, n_0 is the refractive index of the solvent, N_A is Avogadro's number, and λ_0 is the vacuum wavelength of the laser. The excess Rayleigh ratio is the normalized scattering intensity, and given by the following equation for a specific scattering angle:

$$R = \frac{I_A {n_0}^2 R_T}{I_T {n_T}^2} \quad (5)$$

Here, I_A is the excess scattering intensity of the sample, n_0 is the refractive index of the solvent, and R_T, I_T, and n_T are the Rayleigh ratio, scattering intensity, and refractive index, respectively, of toluene, the reference scattering compound.

In the light scattering experiment, the excess scattering intensity is measured, and the Rayleigh ratio for that measurement is calculated based on the various input parameters. For a single protein in solution, the concentration of the protein is adjusted for each measurement, and the Rayleigh ratios computed at each protein concentration are plotted as a function of concentrations to obtain the Zimm plot. The osmotic second virial coefficient and molecular weight of the protein are obtained from the slope and intercept respectively, of the so-obtained linear fit, as defined by equation (3). An example of a Zimm plot is shown in Figure 2.

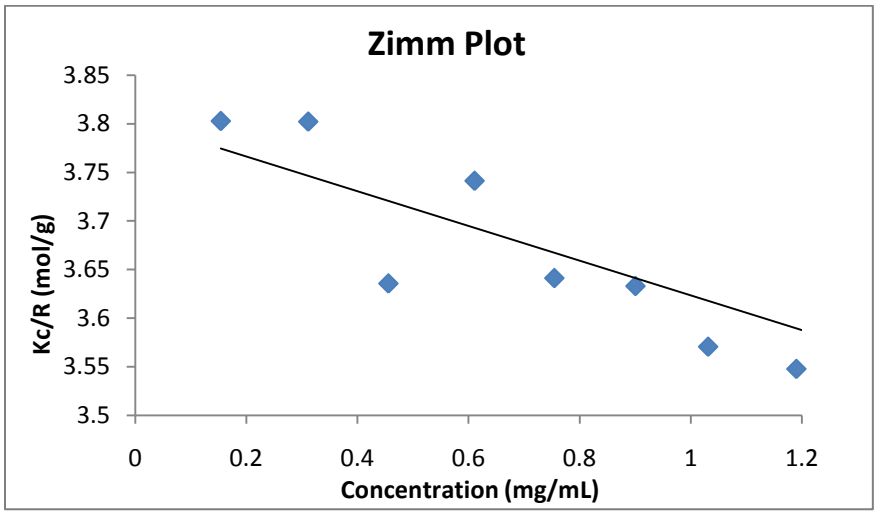

Figure 2: Sample Zimm Plot

When measuring an osmotic second virial cross-coefficient, two scattering species are present in solution, so light scattering from both will contribute to the Rayleigh ratio. Therefore, in this measurement, one component is held at a constant concentration, while the concentration of the other component is adjusted. In our experiments, the polymer (component 2) is held constant as the protein (component 1) concentration is diluted. As

shown by Bloustine et al.,[19] the excess Rayleigh Ratio, (R_{1+2} – R_2), for this two-component system is given by:

$$\frac{Kc_1}{R_{1+2} - R_2} = \alpha + \beta c_1 \quad (6)$$

The excess Rayleigh ratio of the protein/polymer solution relative to the polymer solution is the measured quantity, and α and β depend on osmotic virial coefficients, molecular weights, and differential refractive indices:

$$\alpha = \frac{1}{M_1} + 4mB_{12}c_2 \quad (7)$$

where B_{12} is the osmotic second virial cross-coefficient, and *m* is defined as:

$$m = \frac{M_2(dn/dc)_2}{M_1(dn/dc)_1} \quad (8)$$

and

$$\beta = 2B_{11} + 2[(3C_{112} - 2B_{12}^2M_2) + m(3C_{112} + 2B_{11}B_{12}M_1)]c_2 \quad (9)$$

The data for each cross-interaction experiment corresponds to a single c_2 and consists of a series of R and c_1 measurements. A Zimm plot is constructed from the data, based on equation (6), which yields α and β for that concentration c_2. The experiment is then repeated at different constant concentrations, c_2, to give α and β values over a range of c_2. The α and β are then plotted separately as functions of c_2, and a linear fit is used to obtain the osmotic second virial cross-coefficient from the slope of the α plot, while the β plot is used to find the PEG concentration dependence for protein-protein self-interactions from the intercept.

2. Materials and Methods

Buffer components, bovine chymotrypsinogen-A, and PEG 8000 were all obtained from Sigma-Aldrich. Glassware was washed thoroughly with 5% Contrad 70 detergent, and soaked overnight in 0.5% Helmammex solution. Before use, glassware was rinsed with Barnstead nanopure water; all glassware used for protein or polymer solutions were also rinsed with buffer. Experiments were completed entirely with bis-tris buffer at pH 7, and NaCl was used to set the ionic strength of the buffer to 0.1m (0.11M). The buffer was then stirred and vacuum-filtered through a 0.10 micron PVDF filter.

For the protein self-interaction experiment, a solution of 2 mg/mL bovine chymotrypsinogen-A in buffer was made at room temperature, shaken for 30 minutes, and filtered through a 0.10 micron syringe filter. For the cross-interaction experiments, a PEG 8000 buffer stock was made at the required concentration for each experiment, then stirred and filtered with a 0.10 micron syringe filter. A 10 mg/mL chymotrypsinogen solution was then made using the PEG 8000 buffer, with the same procedure as detailed above for the single-protein solution.

Light scattering measurements were made with a Wyatt mini-Dawn TREOS at a 90° angle and concentrations were measured with a ProStar 325 UV detector from Varian, Inc. After each experiment, the system was primed with a 200 ppm sodium azide and 30% methanol solution for 30 minutes, followed by 30 minutes of 2% Contrad 70, and then

with nanopure water. A Rudolph Research J57 Autotmatic Refractometer, which was calibrated with nanopure water, was used to measure refractive indices.

3. Experimental Design

The methods for the experiments were designed as follows. In all experiments, two injections of pure protein solution were included in a row at the beginning of the experiment to account for adsorption of protein particles onto the system. All replicates were randomized, and each protein injection was separated by two injections of buffer (PEG solution for cross-interaction experiments) to neutralize the system.

For the chymotrypsinogen self-interaction experiment, the measurement concentrations were diluted to 10%, 12%, 14%, 16%, 18%, and 20% protein for three replicates, and to 40%, 60%, 80%, and 100% protein for two replicates. The method is shown in Figure 3 below, with blue peaks indicating concentrations of protein at set times.

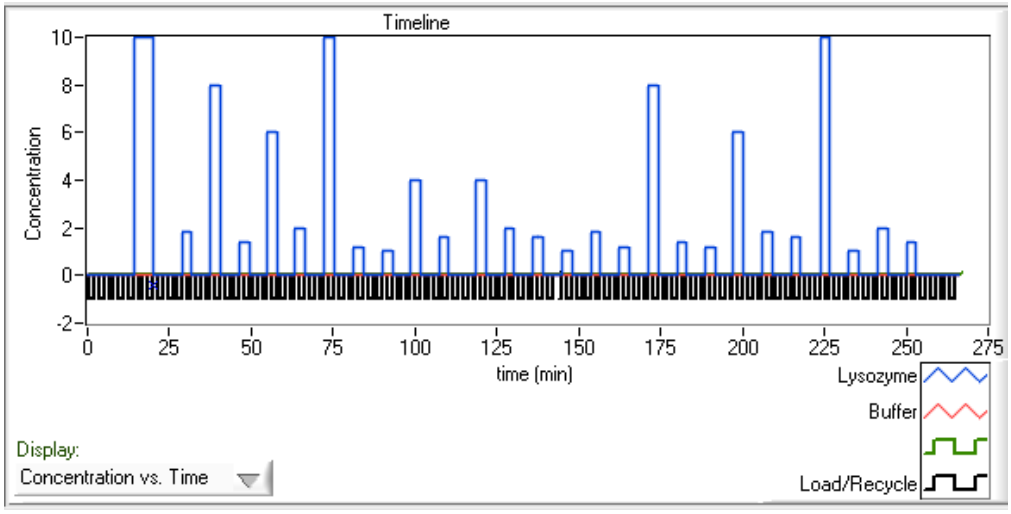

Figure 3: Self-Interaction Method

For the cross-interaction experiments, chymotrypsinogen concentrations of 10%, 20%, 30%, 50%, 70%, and 90% mg/mL were used, with three replicates each. The cross-interaction experiment was repeated at PEG concentrations of 10, 20, 30, and 40 mg/mL. An example of a cross-interaction experiment method is shown in Figure 4. The method was randomized in a different order for each concentration of PEG used.

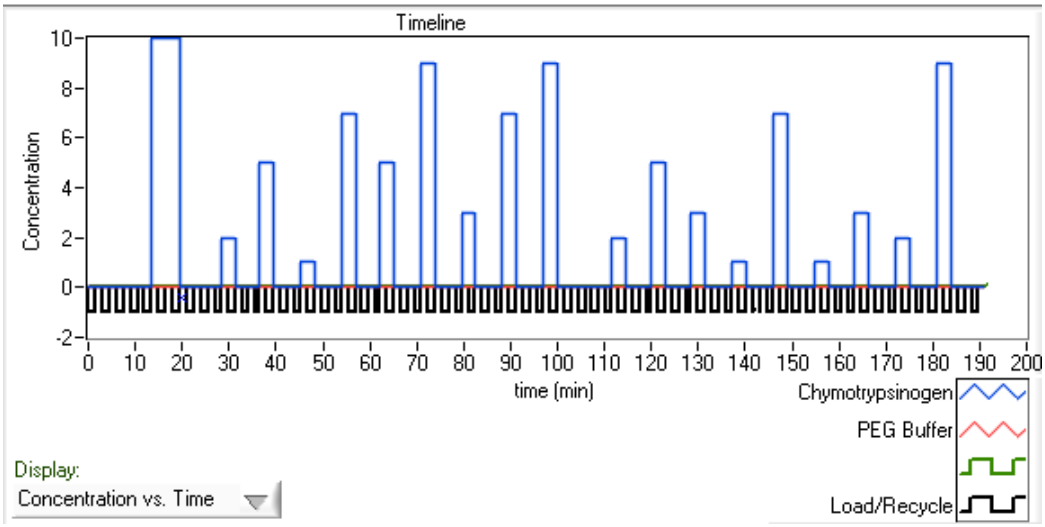

Figure 4: Cross-Interaction Sample Method

In addition, an experiment was completed to measure the differential refractive index of PEG 8000. Measurements of refractive index were taken at concentrations of 0, 10, 20, 30, 40, 50, and 60 mg/mL PEG 8000.

RESULTS AND DISCUSSION

1. Light Scattering Analysis

The results from the light scattering were observed as a set of data peaks similar to the method peaks. Figure 5 gives an example of a close-up of a data peak, with the calibrated light scattering signal in blue and the concentration in black.

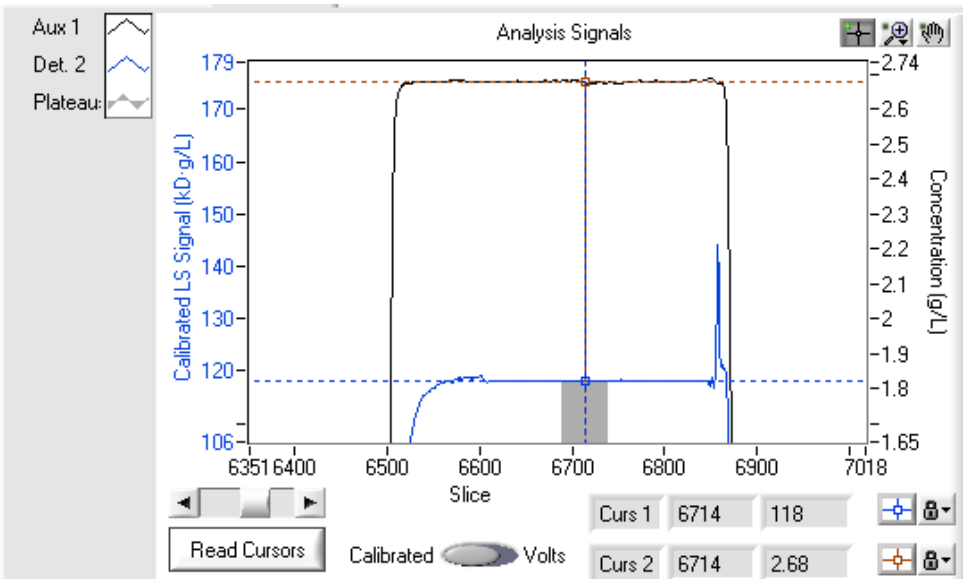

Figure 5: Data Peak

Using the Calypso software, a flat section of each peak was selected with the cursors, and the data were read from the immediate area, marked in grey. The resulting data points were then exported to a text file to be plotted.

2. Results

In order to calculate the cross-interaction coefficient, the parameter *m* (equation 8) is required. This parameter is a ratio of the molecular weights and differential refractive

indices of the two components. The molecular weights are known: chymotrypsinogen is 25670 Daltons and PEG is 8000 Daltons. The differential refractive index, or dn/dc, of chymotrypsinogen is taken to be 0.192 mL/g from literature.[8] The dn/dc of PEG 8000 was calculated by taking measures of the refractive index at different concentrations of PEG. The results are reported in Figure 6. From this plot, the differential refractive index is found to be 0.1254 mL/g. With these values, *m* is calculated to be 0.2035.

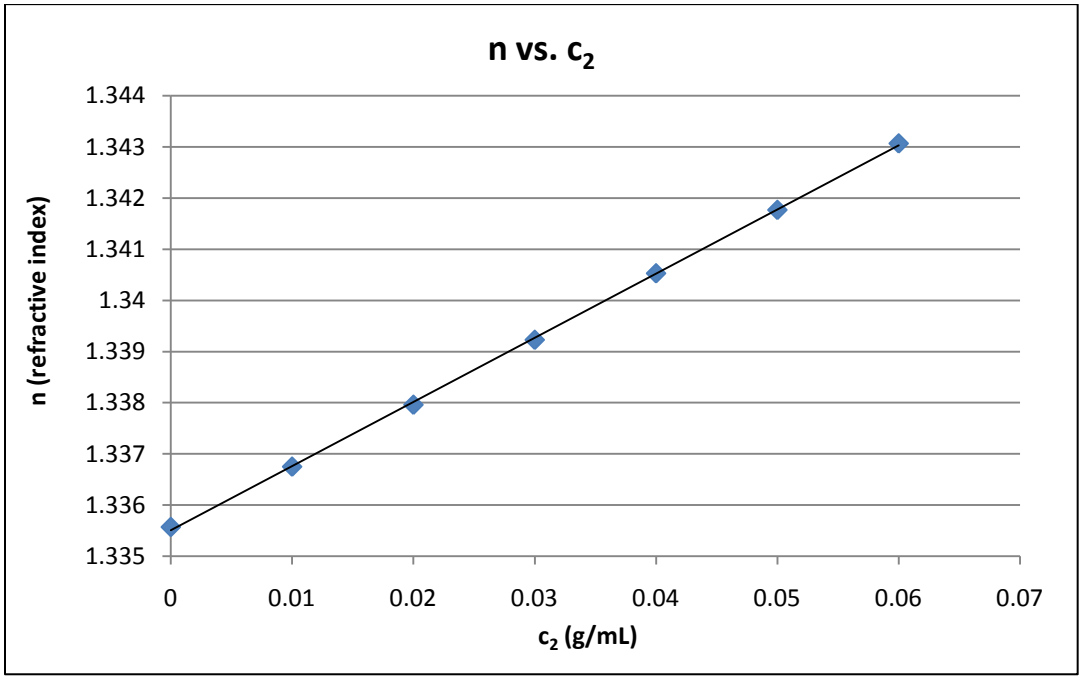

Figure 6: dn/dc of PEG 8000

For each protein concentration, the mean of the replicate measurements was used for the Zimm plot. The mean protein concentration at 10% dilution was used to calculate the real concentration of the stock solution. Values for all concentrations above 1 mg/mL were obtained based on the calculated stock solution concentration. It should be noted

that the UV detector could measure protein concentrations up through 2 mg/mL, but all protein concentrations above 1 mg/mL were recalculated based on the stock solution concentration for consistency.

Systematic deviation in the excess Rayleigh ratios was observed on the Zimm plot at both high and low protein concentrations. These deviations are thought to be due to intrinsic limitations of the light scattering instrument at both high and low concentrations. Therefore, in the linear fits of the data, the highest protein concentration was removed, and only the median values of multiple measurements were used at the lowest concentration. The final result is shown in Figure 7.

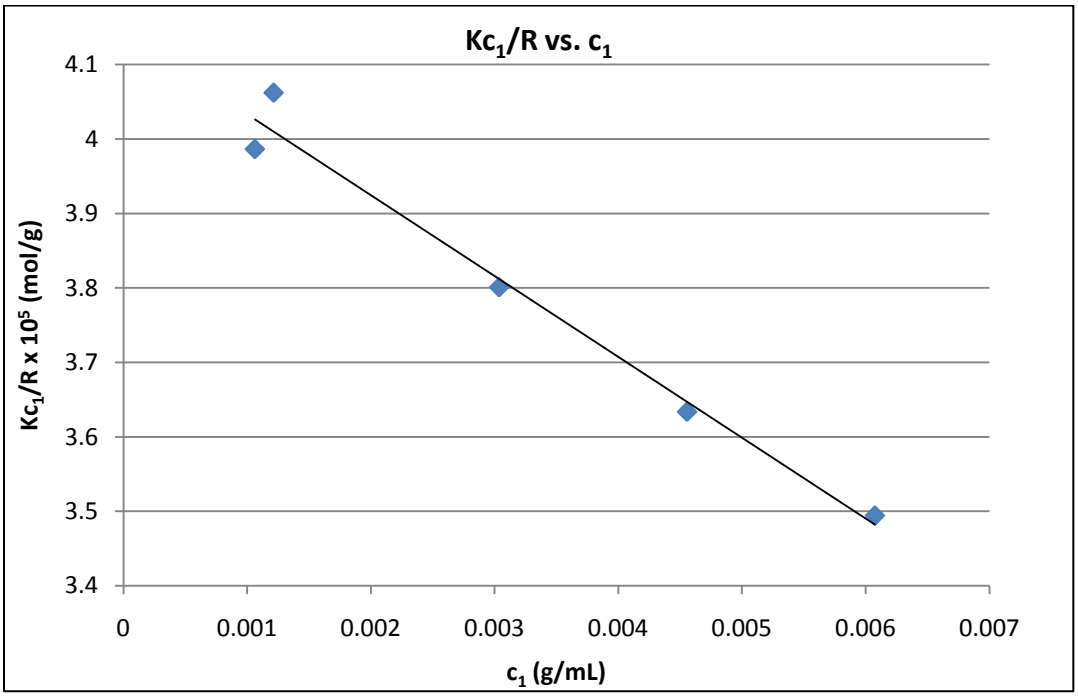

Figure 7: Zimm Plot with c_2 = 0 g/mL, M_1 = 24144 g/mol, B_{11} = -0.00109/2 = -5.43 x 10^{-4} mol.mL/g^2

The linear fit gave an osmotic second virial coefficient of -5.43 x 10^{-4} mol.mL/g^2 for chymotrypsinogen and a protein molecular weight of 24144 Daltons. This molecular weight, rather than the literature value of 25670 Daltons, is used for 0 mg/mL PEG concentration in the determination of the osmotic second virial cross-coefficient for chymotrypsinogen and PEG.

Zimm plots for the protein/PEG solutions at constant PEG concentration, plotted as a function of protein concentration, are given in Figures 8-11.

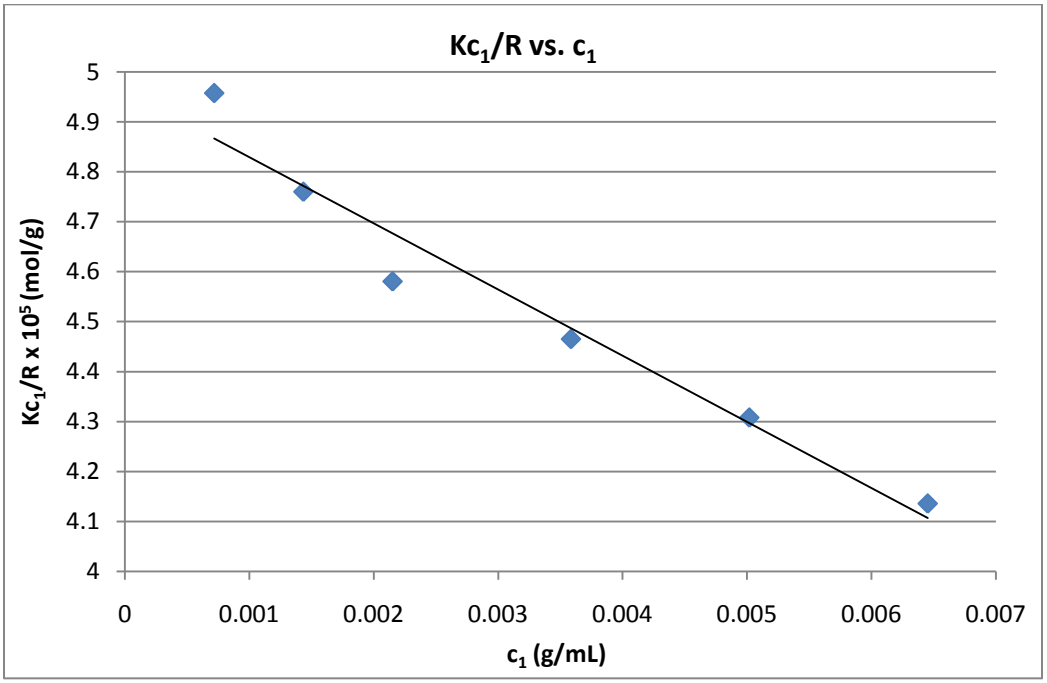

Figure 8: Zimm plot with c_2 = 0.01 g/mL, α = 20154 g/mol, β = -0.00133/2 = -6.63 x 10^{-4} mol.mL/g^2

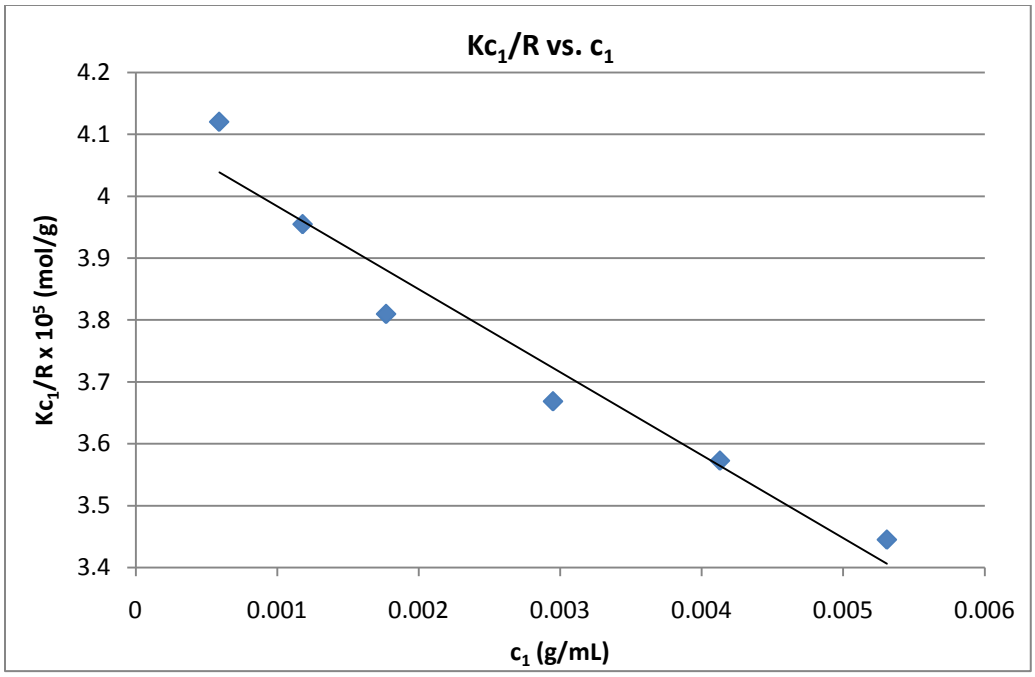

Figure 9: Zimm plot with c_2 = 0.02 g/mL,
α = 24287 g/mol, β = -0.00134/2 = -6.70 x 10^{-4} mol.mL/g^2

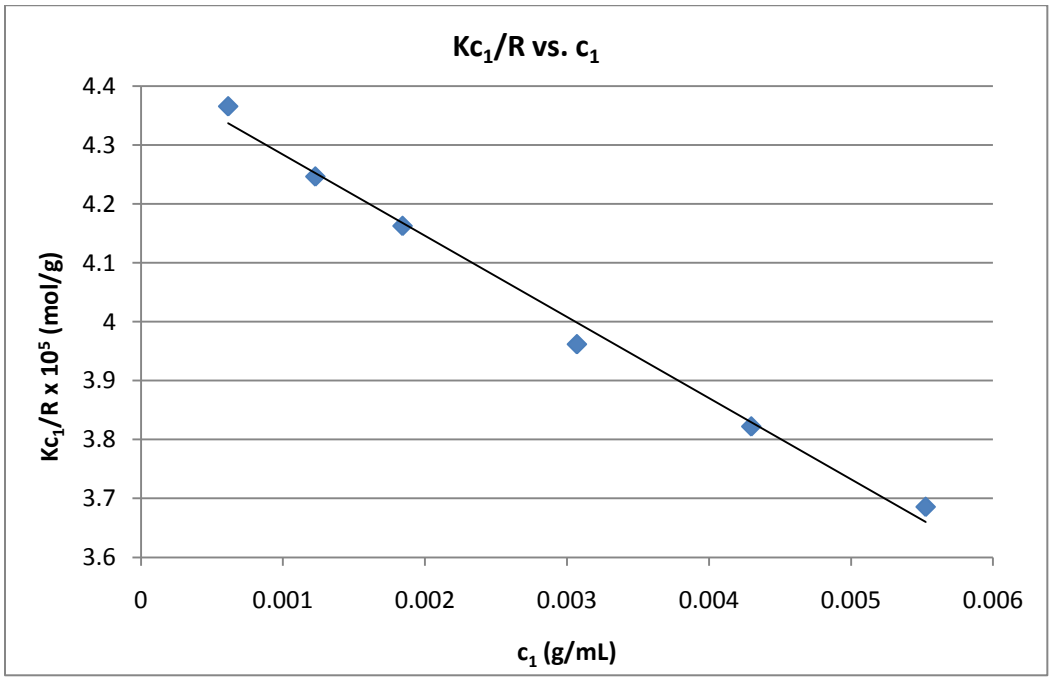

Figure 10: Zimm plot with c_2 = 0.03 g/mL,
α = 22617 g/mol, β = -0.00138/2 = -6.89 x 10^{-4} mol.mL/g^2

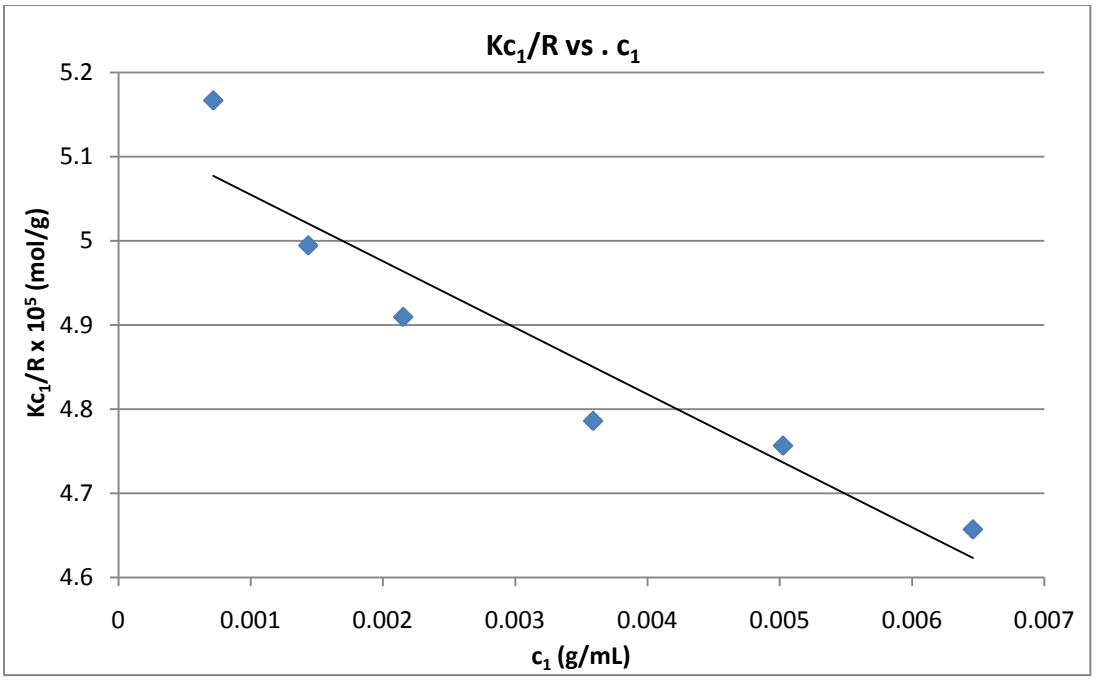

Figure 11: Zimm plot with c_2 = 0.04 g/mL,
α = 19480 g/mol, β = -0.00079/2 = -3.95 x 10^{-4} mol.mL/g^2

The values of α obtained from the Zimm plots are graphed as a function of PEG concentration, c_2, in Figure 12.

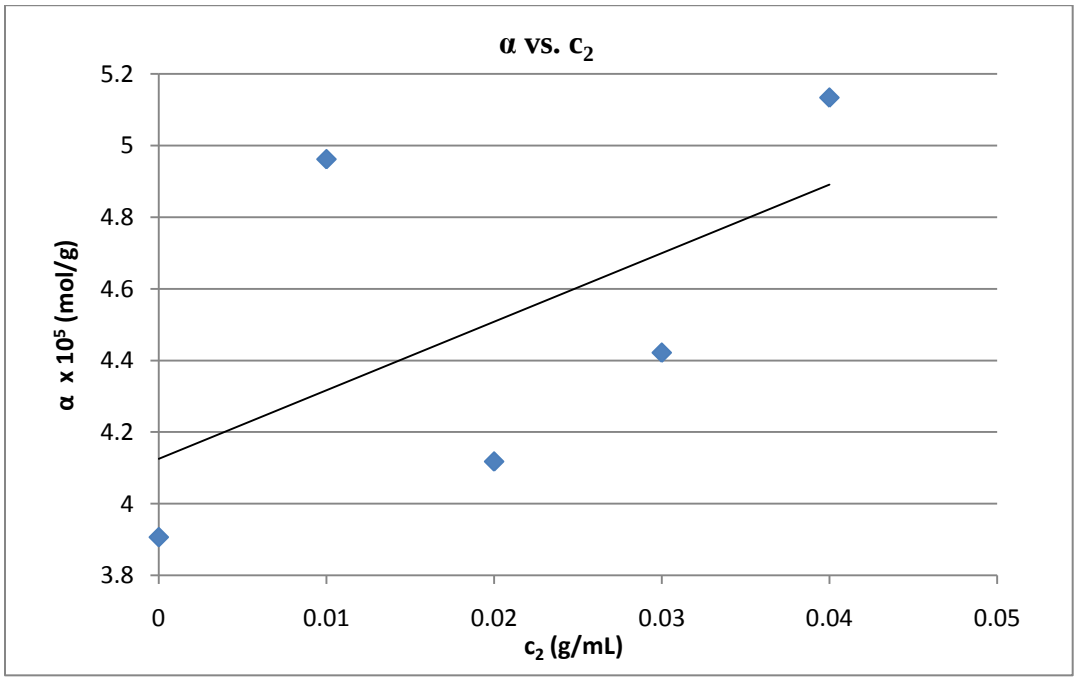

Figure 12: Plot of α as a function of PEG concentration

From this plot and Equation 7:

$\frac{1}{M_1} = 4.13 \times 10^{-5}$ and $4mB_{12} = 0.00019$

which gives a molecular weight of 24241 g/mol for the chymotrypsinogen, compared to the literature value of 25670 and an osmotic second virial cross-coefficient of

$B_{12} = 2.35 \times 10^{-4}\, mol.mL/g^2$.

The β-values obtained from the Zimm plots are graphed as a function of the PEG concentration, shown in Figure 13.

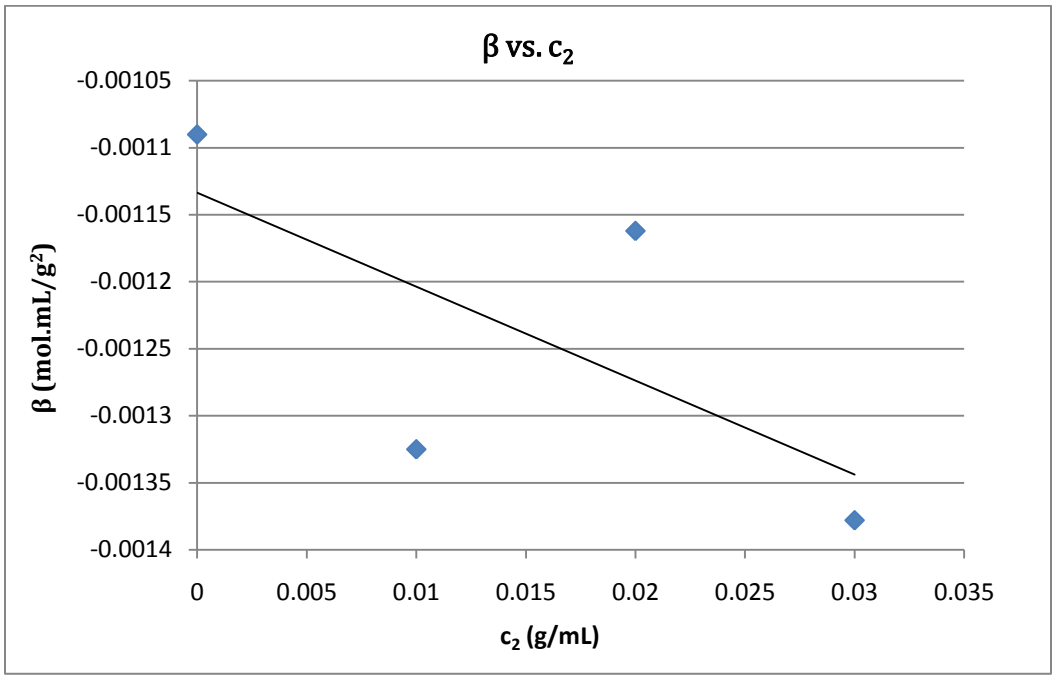

Figure 13: Plot of β as a function of PEG concentration

The intercept of this plot is twice the osmotic second virial coefficient for chymotrypsinogen in the absence of PEG (equation 9): -5.43 x 10^{-4} mol.mL/g^2. The slope gives the C_{112} = -5.46 x 10^{-4} mol.mL^2/g^3, which can be used to calculate the effective osmotic second virial coefficient for chymotrypsinogen in the presence of PEG (equation 10):

$$B_{11}^{eff} = B_{11} + c_2[3C_{112} - 2B_{12}^2 M_2]. \quad (10)$$

The concentration dependence of B_{11}^{eff} is given by,

$$\partial B_{11}^{eff}/\partial c_2 = 3C_{112} - 2B_{12}^2 M_2 = -2.52 \times 10^{-3}\ mol.mL^2/g^3.$$

3. Discussion and Conclusions

The second virial coefficient obtained in the self-interaction experiment, which was done at pH 7 and ionic strength 0.11M, is -5.43 x 10^{-4} mol.mL/g^2. This is comparable to the study done by Velev et al.[8], wherein the second virial coefficient at pH 6.8 and ionic strength 0.1M was found with static light scattering to be -4.10 x 10^{-4} mol.mL/g^2. In the same study, the molecular weight was obtained to be 24450 Daltons from light scattering, and reports a literature value of 23660 and a calculated value of 25670 Daltons. Comparatively, the experimental molecular weight is 24144 Daltons, which is close to the value obtained in the Velev study.

The cross-interaction coefficient is 2.35 x 10^{-4} mol.mL/g^2, which shows an extremely slight positive interaction, indicating that the protein and polymer have little interaction beyond a small amount of repulsion. Bloustine et al.[20] report a value of 1.7 x 10^{-4} mol.mL/g^2 for the cross-interaction of PEG 8000 and lysozyme at pH 6.2 and ionic strength 0.5M. Although their experiment is not completely comparable, due to the differences in buffer conditions and, more importantly, protein type, the general trend observed is similar. The self-interaction of lysozyme at the conditions in the Bloustine paper is given as approximately -4 x 10^{-4} mol.mL/g^2, or slightly self-attracting, and adding PEG 8000 to this protein causes a small positive cross-interaction.

In addition, the concentration dependence of B_{11}^{eff} is -2.52 x 10^{-3} mol.mL^2/g^3. From the study by Bloustine et al.,[19] when the concentration dependence is negative, PEG induces

attraction between the protein molecules. This increased self-attraction is most likely due to depletion theory, which states that the addition of PEG increases protein self-attraction, as PEG promotes protein stability. It should be noted that the Bloustine study found decreasing protein attraction when adding PEG 8000 to lysozyme. Therefore, it can be theorized that the addition of PEG 8000 to a self-attractive protein causes the protein to become less self-attractive.

In conclusion, this study has found a trend that adding PEG 8000 to chymotrypsinogen causes a slight amount of repulsion between the two components, and increases the chymotrypsinogen's attraction to itself. Based on the paper by Bloustine et al.[19], it is suggested that the addition of PEG 8000 to slightly self-attractive proteins results in very minimal repulsive protein-polymer interactions. Due to the large scattering present in the plots for α and β, it is recommended that the experiments be repeated to confirm the trend.

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APPENDIX

c_2 (g/mL)	n (refractive index)
0	1.33557
0.01	1.33675
0.02	1.33796
0.03	1.33923
0.04	1.34053
0.05	1.34177
0.06	1.34307

Table 1: dn/dc data

c_1 (g/mL)	$Kc_1/R \times 10^5$ (mol/g)
0.001063	3.986449
0.001215	4.062201
0.003038	3.80077
0.004556	3.633475
0.006075	3.494005

Table 2: Chymotrypsinogen Self-Interaction Data, PEG = 0 g/mL

c_2 = 0.01 g/mL	
c_1 (g/mL)	**$Kc_1/R \times 10^5$ (mol/g)**
0.000717	4.957833
0.001434	4.760175
0.002151	4.580553
0.003585	4.465066
0.005019	4.30782
0.006453	4.135572
c_2=0.02 g/mL	
0.00059	4.120225
0.00118	3.954918
0.00177	3.809855
0.00295	3.668771
0.00413	3.5729
0.005309	3.445319
c_2=0.03 g/mL	
0.000614	4.365648
0.001228	4.246441
0.001842	4.162559
0.00307	3.961732
0.004299	3.822078
0.005527	3.68558
c_2=0.04 g/mL	
0.000718	5.166866
0.001436	4.99454
0.002153	4.909591
0.003589	4.786064
0.005024	4.756665
0.00646	4.657164

Table 3: Data for Cross-Interaction Experiments

c_2 (g/mL)	$\alpha \times 10^5$ (mol/g)
0	3.90625
0.01	4.9616
0.02	4.1175
0.03	4.4214
0.04	5.1336

Table 4: α as a function of c_2

c_2 (g/mL)	β (mol.mL/g^2)
0	-0.00109
0.01	-0.001325
0.02	-0.001162
0.03	-0.001378

Table 5: β as a function of c_2

n_2 (mL/g)	B_{11}, (mol.mL/g^2)	M_1, self interaction (g/mol)	B_{12} (mol.mL/g^2)	C112, (mol.mL^2/g^3)	$\partial B_{11}^{eff}/\partial c_2$ (mol.mL^2/g^3)
0.1254	-5.43×10^{-4}	24144	2.35×10^{-4}	-5.46×10^{-4}	-2.52×10^{-3}

Table 6: Summary of Results